Evaluation of recombinant leucine aminopeptidase as vaccine antigen against *opisthorchis viverrini* infection

Jittiyawadee Sripa[1*], Banchob Sripa[2], Thewarach Laha[3]

[1]College of Medicine and Public Health, Ubon Ratchathani University, Thailand

[2]Tropical Disease Research Laboratory, Department of Pathology, Khon Kaen University, Thailand

[3]Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand

Abstract

Leucine aminopeptidase, the methalloprotease of liver fluke, *Opisthorchis viverrini* (*Ov*LAP) was produced as active recombinant protein in bacteria and tested potential as vaccine in strategy of decrease infection. Active r*Ov*LAP was emulsified with aluminium hydroxide and injected to syrian golden hamsters in the test group (5 hamsters) via subcutaneous route. The control groups were injected with aluminium hydroxide and aluminium hydroxide emulsified with elution buffer, respectively. Infection was induced by orally with *O. viverrini* metacercariae after complete course of injection. High level of humoral immunity of hamster including IgM, IgA and IgG was detected in test group. This immunity was present in serum and persisted in high level throughout course of experiment. Inducted immunity, IgM, IgG and IgA were also present in bile but those antibodies level were detected in lower level than in serum. In test group, partial protection was observed with 20.77% of worm reduction ($P<0.05$) but increase of egg production per worm ($P<0.05$) was found. r*Ov*LAP is highly immunogenicity that indicated as good antigen for induction host immunity. Further studies on r*Ov*LAP as drug target/vaccine are needed to perform. Combine this molecule with other vital predominate molecules or specify local region of induction immunity are needed to considered to further development.

Key words: *Opisthorchis viverrini*, Leucine aminopeptidase, Vaccine

Introduction

Opisthorchiasis and cholangiocarcinoma that caused by *Opisthorchis viverrini* are still the problem in Southeast Asians countries, especially in Northeast of Thailand (Sripa et al., 2007). Due to this problem, the various extensive strategies have been performing to control opisthorchiasis in human host and mammalian host

reservoirs. Although anti-helminthic drug, praziquantel is widely use in aspect of eliminate opisthorchiasis but drug resistance and some adverse effects has been report (van den Enden, 2009). However, tribendimidine has been developed and seems to be efficient for *O. viverrini* treatment but some adverse effects and limitation of drug usage in levamisole and pyrantel resistance strains has been report (Hu, Xiao, and Aroian, 2009; Soukhathammavong et al., 2011). To avoid drug resistance and adverse effect from drug parasite treatment, vaccine and new target of drug action has been attractive to study.

Leucine aminopeptidase (LAP) is the attractive molecule for induction host immunity against helminthic infection. Recombinant LAP protein was proposed as immunogenic molecule that elicits protective Th1 and Th2 humoral immune response in mice infected with *Fasciola gigantica*. This inducted immunity was significantly decrease worm recovery in infected mice (Changklungmoa et al., 2013). Moreover, another report was showed significantly decrease *F. hepatica* recovery from sheep after induction of humoral protective immunity with recombinant LAP protein (Maggioli et al., 2011).

In contrast of opisthorchiasis, protective immunity was occurred during the course of infection and involved in worm elimination at re-infection. (Sirisinha et al., 1983a; Sirisinha et al., 1983b). This protective immunity was decreased 30% of worm recovery after infected hamsters with a small number of flukes prior to single immunization with aqueous somatic extract of adult worms (Wongratanacheewin et al., 2003). Although, vaccination with parasite crude extract and induced immunity by infected with specific parasite were showed high number of worm reduction, but due to the safety reasons, vaccination with specific antigen is recommended. At any rate, there is no report of protective immunity that induction with specific antigen in opisthorchiasis animal model. In the day of the recombinant technologies are available, the recombinant vaccine seem to be more attractive for induction host immunity with reasons of safe, stable and easy to manipulation.

In addition, accomplishment of using recombinant protein including LAP in vaccination against other helminthic infection, thus the effective vaccination with this molecule could be possible in opisthorchiasis hamster model. Therefore, in this study, *O. viverrini* leucine aminopeptidase (*Ov*LAP) that present in all stages of worm, high

immunogenicity and play role as gut-associated enzyme was produced as recombinant protein and assess vaccine efficacy against *O. viverrini* infection in hamster model. The protective efficacy of this vaccine was evaluated in terms of parasitological parameters including worm burden and egg production.

Materials and methods

1. Animals

Male Syrian golden hamsters, aged 6-8 weeks obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University were used in this experiment. The hamsters were divided into 3 groups (5 hamsters per group) for evaluated vaccine trial. All animals ethical and procedures were approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand, Record No. AEKKU 13/2557.

2. Antigen preparation

Purified recombinant *O. viverrini* leucine aminopeptidases (r*Ov*LAP) was prepared to vaccinate into hamsters. Briefly, full length of *Ov*LAP was amplified from adult *O. viverrini* cDNA library with specific primers incorporated with NdeI endonucleases (in bold) (Thermoscientific, USA) and added with stop codon in reverse primer (in bold and underlined). The forward primer was 5' GCG GCG **CAT ATG** ATG TCT GTG AGC CGT AGC GTC3' and reverse primer was 5' GCG GCG **CAT ATG** **<u>TCA</u>** CAG TTT GGA AAC CAC CTC 3'. The amplification reaction was performed and DNA fragment was recovered, purified, cloning into TOPO-T vector (Invitrogen, USA) and then sub-cloning into expression vector, pET 15b+ (Novagen, USA). The recombinant protein of *Ov*LAP was produced in bacterial host, BL21(DE3) and induction for protein expression with 1 mM IPTG at 26 $^{\circ}$C for 8 hrs at a shaking speed of 225 rpm. Then, the bacterial pellet was collected and the supernatant was discarded before re-suspended the pellet with 3 ml native condition binding buffer containing 1X cocktailed proteases inhibitor (without EDTA) (Sigma-Aldrich, USA). The re-suspended pellet was frozen and thawed and then subsequently to sonication. The pellet debris was removed by centrifugation and the supernatant containing r*Ov*LAP protein was collect for protein purification through Ni-NTA resin column (Thermoscientific, USA). Purified protein was analyzed with SDS-PAGE to

observing a single band with coomassie blue and/or protein immunoblot staining with His-Tag antibody. The r*Ov*LAP protein were pooled and concentrated by concentrator (Eppendrof concentrator 5301). The concentrated protein was analyzed by SDS-PAGE and the protein concentration was determined its absorbance at 280 nm (Nanodrop, Thermoscientific, USA).

3. Vaccination

The hamsters were divided into 3 groups, 5 hamsters each, including one test group and two control groups. Each group was injected as the details in table 1. Briefly, the test group were vaccinated with 100 ug in volume of 100 ul of r*Ov*LAP protein formulated with equal volume of Alum Adjuvant. In other control groups were vaccinated with native elution buffer formulated with Alum Adjuvant and Alum Adjuvant alone (Imject^{TM} Alum Adjuvant, Thermoscientific, USA), respectively. All three groups were injected subcutaneously for three times, two weeks interval. Before each injection, the peripheral blood samples were collected from hamsters by retro-orbital eye bleeding. One week after the third injection, all hamsters were challenged infection with 50 metacercariae orally.

4. Determination of worm egg count and worm recovery

At one month after challenged infection, the hamsters were separated to one per cage for feces collection. The feces were collected a week interval for 9 weeks to determined the number of *O. viverrini* egg per gram (EPG) with the quantitative formalin/ethyl acetate concentration technique (Elkins, Haswell-Elkins, and Anderson, 1986). The worm burdens were determined at the day of sacrificed the hamster by counting worms in the liver squashes and hamster feces in intestine were removed for determined *O. viverrini* egg. To determine the egg production per worm, 10 adult worms from each vaccinated group were collected and washed with physiological saline. The worms were homogenized and the numbers of eggs in the uterus were counted under the microscope.

5. Detection of hamster antibody response by ELISA

Pre and post-immunization sera were collected by retro-orbital eye bleeding to determine antibody level against r*Ov*LAP using indirect ELISA. At the day of sacrificed hamsters, blood samples were bled from the heart. All blood samples were

spin at 3000-3,500 rpm at 4 ^{o}C to collected hamster sera. All sera were kept at -20 ^{o}C until testing.

Pre and post immunization sera from hamsters were tested individually by ELISA for the presence of anti-r*Ov*LAP antibody in hamsters from all three groups. For total IgG antibody detection, 1 ug/ml of r*Ov*LAP protein was coated on 96 well-plate (Nunc Maxi-Sorp Immuno Plate, Roskide, Denmark) and incubated for overnight at 4 ^{o}C. Plates were washed and antigen was blocked with 5% skimmilk and incubated at 37 ^{o}C for 2 h. One hundred ul of 1:1000 hamster serum was added and incubated at 37 ^{o}C for 2 h. Plate was washed again and then HRP conjugated anti-hamster IgG (Zymed) at dilution 1:10,000 were added and for 1 h at 37^{o}C. Plate was washed again and freshly prepared OPD substrate solution (1 tablet Zymed in 12 ml of Citrate-phosphate buffer, pH 5.0) was added and incubated for 30 minutes at 37^{o}C. The reaction was stopped by adding 0.5 M sulfuric acid (H_2SO_4) 100µl/well. The ODs were measure on an ELISA reader (TECAN, Austria) at OD 492 nm. Each sample was always assays in duplicate. Each plate included blank (non-coated antigen). For detection of IgM and IgA antibody, all procedures were similar with total IgG antibody detection but the concentration of coated antigen, serum dilution and secondary antibody dilution were changed. 2 ug/ml of r*Ov*LAP protein were coated on 96 well-plate for detecting IgM and IgA antibody against r*Ov*LAP protein. Hamster serum at dilution 1:100 and secondary antibody against IgM and IgA anti-hamster at dilution 1:1,000 were use in measured of IgM and IgA antibody to r*Ov*LAP protein. To measure the antibody level of each sample, the OD values were subtracted from the mean OD of blank in each ELISA plate. Inducted IgM, IgG and IgA was also detect in bile with the same procedure.

6. Statistic analysis

The worm burden, EPG and egg per worm data, antibody level in each group was determined to examine the effects of r*Ov*LAP protein vaccination against *O. viverrini* infection. All statistic analysis was analyzed using GraphPad Prism 5.

Results

1. Total IgG, IgM and IgA levels against r*Ov*LAP protein in hamster

Serum samples from hamsters were tested for the presence of the total IgG, IgM and IgA level against r*Ov*LAP protein by using ELISA (Figure 1). The individual

serum after first, second immunization and after sacrificed hamsters were tested and calculated for mean OD ± SD at 492 nm.

In the group of vaccination with r*Ov*LAP protein, the levels of IgM and IgA antibody against r*Ov*LAP protein were increased rapidly after first vaccinations. In contrast with total IgG antibody level that was slightly increases. In addition, the antibody level of IgM and IgA antibody was also maintained in significant higher level than total IgG antibody. However, IgM antibody level was slightly decreased at the day of sacrificed hamster (week 10 after final vaccination). While, IgA antibody against r*Ov*LAP protein was maintained over the time course of the duration of the experiment. The total IgG, IgM and IgA level of pre-immunized sera and sera from negative control group (immunized with alum adjuvant alone and alum adjuvant formulated with elution buffer) were not raised against r*Ov*LAP protein (data not shown).

In bile, there was detected some of level IgM, IgG and IgA. However, the level of those antibodies was lower than detected in serum (Figure 1).

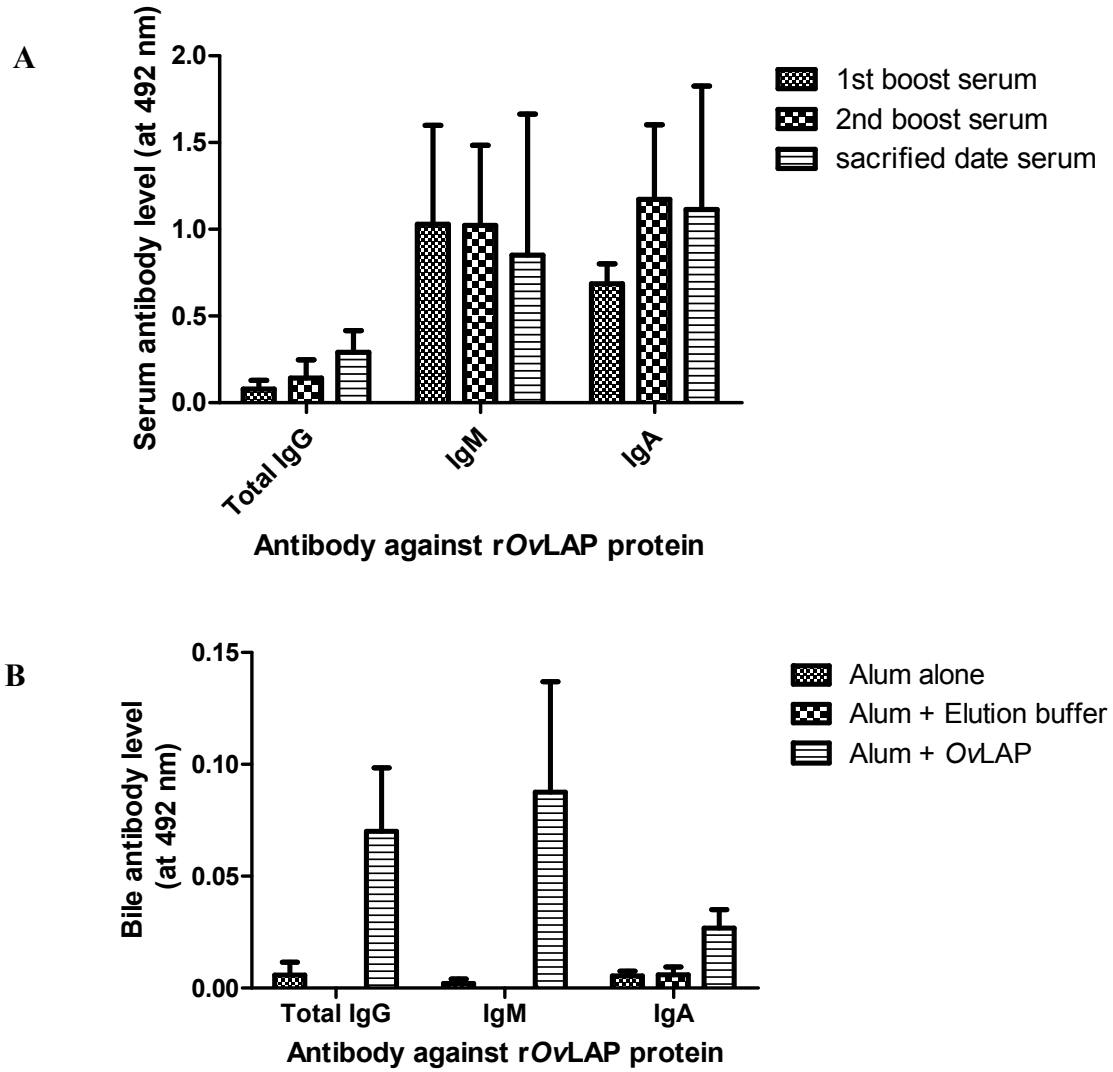

Figure 1 Total IgG, IgM and IgA antibody level against r*Ov*LAP protein in hamster after vaccination. In hamster serum, IgM antibody was showed increase rapidly after first vaccination. While IgA antibody still maintained in high level until week 10 after final vaccination but IgM antibody was slightly decrease. In contrast of total IgG antibody that slightly increases with the lower level than IgM and IgA antibody (A). In bile of hamster, total IgG, IgM and IgA were detected in bile but lower than detected in serum (B).

2. Reduction of worm burden and egg production in vaccinated hamsters

The protection after challenge infection in vaccinated hamsters was determined from egg production rate and worm burden. For egg production rate, feacal egg count was determined in hamster feces for 9 weeks after challenge infection for one month. Egg production of vaccinated hamsters was present as egg per gram (EPG) of feces material. The EPG of all vaccinated hamster groups were gradually increased from week 1 to week 9 of feacal collection schedule. The variation of egg production rate was observed in all three groups of hamster. The difference of mean egg counts between the test group (vaccinated with r*Ov*LAP protein) and the control group (vaccinated with native elution buffer and alum adjuvant alone) was not significant ($P>0.05$) (Figure 2).

Number of egg production per worm was also determined in all three groups of vaccinated hamster. Worms from hamsters that vaccinated with r*Ov*LAP protein was present high rate of egg production. In contrast with control group that presented lower number of egg production per worm. However, there was not significantly different of egg production per worm among three group ($P<0.05$) (Table 1). The average of worm burden in each group of vaccination was determined at the day of sacrificed hamsters. The insignificant number of worm reduction of the test group was observed. The percent of worm reduction in test group was 20.77% when compared with control group ($P<0.05$).

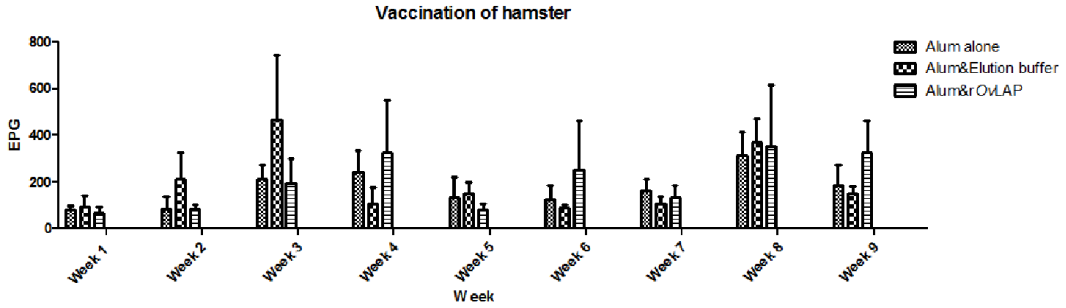

Figure 2 Quantitative fecal egg counts in feces from experimental hamsters that vaccination with r*Ov*LAP protein, elution buffer and alum adjuvant alone. The number of egg count in feces was determined as weekly interval after one month of *O. viverrini* challenged infection with 50 metacercariae. No significant difference was found among number of EPG in three vaccinated group ($P<0.05$). The data of EPG was presented as mean ± SD.

Table 1 Details of vaccination and protection against *O. viverrini* in hamsters vaccinated with r*Ov*LAP protein, elution buffer and alum adjuvant alone.

Group No. 5 hamster/group	Antigen	Route	Dose	Adjuvant	Mean worm burden ± SD	%reduction in worm burdens (*P*-value)	Mean EPG/worm (*P*-value)
1	r*Ov*LAP protein	Subcutaneous	100 ug	Alum adjuvant	25.75 ± 4.856	20.77% ($P<0.05$)	4536 ($P<0.05$)
2	Elution buffer	Subcutaneous	100 ul	Alum adjuvant	32.50 ± 9.147	-	2942 ($P<0.05$)
3	Alum adjuvant	Subcutaneous	100 ul	-	25.80 ± 4.087	-	4490 ($P<0.05$)

Discussion

Leucine aminopeptidases (LAP) has been proposed as the important molecule in biological functions of all organisms (Matsui, Fowler, and Walling, 2006). In helminth, role of LAP in various tasks has been recognized. Gut-associated enzyme also has mentioned as the role of LAP in helminth (McCarthy et al., 2004). The role in egg hatching also report, since decrease of egg hatching was found after knockdown both two discrete of SmLAP genes (Rinaldi et al., 2009). In addition, recombinant LAP was also tested as vaccine potential. Immunity against r*Fh*LAP and r*Fg*LAP were found significant reduction of worm burden (Changklungmoa et al., 2013; Maggioli et al., 2011).

In *O. viverrini*, one discrete of LAP (*Ov*LAP) was found and characterized. Basic features of *Ov*LAP including primary sequence and basic mechanism are corresponds with other helminthic LAP. High immunogenicity was also found in r*Ov*LAP, similar with r*Fh*LAP and r*Fg*LAP. Ability as immunogen and high protection from r*Fh*LAP and r*Fg*LAP vaccination arouse r*Ov*LAP was attractive for tested potential as vaccine against *O. viverrini* infection. Hamsters were vaccinated with r*Ov*LAP via subcutaneous route to induce antibodies production in serum and/or mucosa to block/neutralize/interfere parasite that following infection after full course of injection. The quantity of inducted antibodies including IgM, IgG and IgA was determined in serum and bile using ELISA for convenience and rapidity. Highly increase of immunoglobulin, especially subtype M and A was detected in serum after first vaccination. In contrast with detection in bile that low level of total IgG, IgM and IgA was detected. However, IgG, the important antibody for protection was slightly increased in serum and some of IgG level was detected in bile. Some of those antibodies were diffused from blood vessel to gall bladder and interact with specific region or interrupt function of specific molecule in worm by pass through their mouth or tegument. This interaction may cause parasite death but no effect on parasite reproductive organ since high EPG was determined.

Although, humoral inducted immunity was persisted for almost three months, but few of immunity were diffused into worm habitat. However, some of highly inducted immunity, IgG, IgM and IgA was estimate to diffuse along host's mucosal surface of gastrointestinal tract and could contact with newly excysted larva that crawl

up to their habitat in secondary bile duct. Moreover, inducted immunity against r*Ov*LAP was estimate to effect on various stages of *O. viverrini* since the expression of *Ov*LAP was found throughout their life cycle. However, specific location of inducted immunity production still doubt, but recently, oral vaccine has been established to specify local region of immunity induction to reduction of colonization and protection mucosal region from pathogen. Spore of *Bacillus subtilis* was used as vehicle to deliver vaccine via oral route. Surface of *B. subtilis* spore has been design for expressing specific protein to interact and induce host immune response after feed via oral. Success of induction of high level of local mucosal immunity both sIgA and secreting IgA cells was found after feed animal with *B. subtilis* spore expressing *C. sinensis* LAP (*Cs*LAP) on their surface (Yu et al., 2015). In case of *O. viverrini* that contact with mucosa of host gastrointestinal tract, induction host immunity by orally with spore expressing protein on their surface is attractive to be considered to develop as drug/vaccine against *O. viverrini* infection.

However, inducted immunity against r*Ov*LAP can decrease 20% of worm recovery compared with control groups. The percent of worm reduction was quite similar to vaccination with crude *O. viverrini* metacercarial extract that can reduce 21% of worm burden. In contrast of r*Ov*-CB-1 that can reduce only 18% (Unpublished). This showed that single active molecule of r*Ov*LAP has ability to produce protective immunity against *O. viverrini* infection in similar level of multi-antigenic molecule in metacercarial extracts. Since safe vaccine has been concern, ability of induction effective and specific immunity with low adverse effect from excess antibodies in circulation of single active molecule was attractive than multi-antigenic molecule. Thus, r*Ov*LAP is the first identified molecule of *O. viverrini* that was fascinated to further develop as drug/vaccine target.

However, *O. viverrini* is complex organism that has specialized structure and molecules performing in multi tasks. Several molecules of *O. viverrini* have more than one isoforms that work sequential and concert manner. Inhibition one of important molecule could not breakdown biological process of worm since relevant molecule has been recruited to play their related role. Interruption of predominate vital molecules or relevant molecules would have impact on *O. viverrini* survival. Several molecules of *O. viverrini* has been found vital for maintain their life cycle

(Smout et al., 2009; Suttiprapa et al., 2009). Combination of these vital molecules would be efficient to breakdown parasite life cycle. Moreover, vital molecules that derived from different stages of the parasite's life cycle or different species of the parasite would be elicit a strong immune response and attractive to evaluate for vaccine against *O. viverrini* infection. Similar in previous studies that demonstrated high efficiency of hybrid or multivalent recombinant protein vaccination in infectious disease such as fascioliasis (Jayaraj et al., 2009), schistosomiasis (Chen et al., 2005), malaria (Li et al., 1999), and leishmaniasis (Coler et al., 2002).

Moreover, helminths are long-lived organisms in specific host because they use antigenic variation to escape from the hosts' immune attack. In addition they are developed different strategies for survival in their human host. For example schistosomula can compromise complement function (Ouaissi et al., 1981) and degrade host immunoglobulins (Auriault et al., 1981). Thus, host immune response is not enough to eradicate the parasite. Vaccination is the method to increase host immunity to destroy and eradicate parasite from their host. The highly induction of specific host immune response against the parasite is important for worm elimination. Helminth infections are typically associated with hypereosinophilia, considerable IgE production, mucous mastocytosis, and goblet cells hyperplasia. The vaccinations that can induce these mechanisms may have potential to reduce parasitic infection because these immune parameters are involved in different effector mechanism to worm elimination. Moreover, the combination of the function of humoral and cell mediated host immune response may also increase the reduction of worm and decrease worm egg production (MacDonald, Araujo, and Pearce, 2002). The appropriate induction of immune response mechanisms may allow the rational development of more efficacious *O. viverrivi* vaccine.

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References

Auriault, C., Ouaissi, M. A., Torpier, G., Eisen, H., and Capron, A. (1981). Proteolytic cleavage of IgG bound to the Fc receptor of *Schistosoma mansoni* schistosomula. *Parasite Immunol* **3**(1)**,** 33-44.

Changklungmoa, N., Kueakhai, P., Riengrojpitak, S., Chaithirayanon, K., Chaichanasak, P., Preyavichyapugdee, N., Chantree, P., Sansri, V., Itagaki, T., and Sobhon, P. (2013). Immunization with recombinant leucine aminopeptidase showed protection against *Fasciola gigantica* in mice. *Parasitol Res* **112**(10)**,** 3653-9.

Chen, Y. X., Wang, L. X., Tang, L. F., Zhang, S. K., Zhang, J., Zeng, X. F., and Yi, X. Y. (2005). [Boost effect of recombinant IL-4 on protection of *Schistosoma japonicum* cathepsin B DNA vaccine in mice against the parasite]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **23**(2)**,** 65-8.

Coler, R. N., Skeiky, Y. A., Bernards, K., Greeson, K., Carter, D., Cornellison, C. D., Modabber, F., Campos-Neto, A., and Reed, S. G. (2002). Immunization with a polyprotein vaccine consisting of the T-Cell antigens thiol-specific antioxidant, *Leishmania major* stress-inducible protein 1, and Leishmania elongation initiation factor protects against leishmaniasis. *Infect Immun* **70**(8)**,** 4215-25.

Elkins, D. B., Haswell-Elkins, M., and Anderson, R. M. (1986). The epidemiology and control of intestinal helminths in the Pulicat Lake region of Southern India. I. Study design and pre- and post-treatment observations on *Ascaris lumbricoides* infection. *Trans R Soc Trop Med Hyg* **80**(5)**,** 774-92.

Hu, Y., Xiao, S. H., and Aroian, R. V. (2009). The new anthelmintic tribendimidine is an L-type (levamisole and pyrantel) nicotinic acetylcholine receptor agonist. *PLoS Negl Trop Dis* **3**(8)**,** e499.

Jayaraj, R., Piedrafita, D., Dynon, K., Grams, R., Spithill, T. W., and Smooker, P. M. (2009). Vaccination against fasciolosis by a multivalent vaccine of stage-specific antigens. *Vet Parasitol* **160**(3-4)**,** 230-6.

Li, M., Bi, H., Dong, W., Xu, W., Li, Q., and Li, Y. (1999). A recombinant multi-epitope, multi-stage malaria vaccine candidate expressed in *Escherichia coli*. *Chin Med J (Engl)* **112**(8)**,** 691-7.

MacDonald, A. S., Araujo, M. I., and Pearce, E. J. (2002). Immunology of parasitic helminth infections. *Infect Immun* **70**(2)**,** 427-33.

Maggioli, G., Acosta, D., Silveira, F., Rossi, S., Giacaman, S., Basika, T., Gayo, V., Rosadilla, D., Roche, L., Tort, J., and Carmona, C. (2011). The recombinant gut-associated M17 leucine aminopeptidase in combination with different adjuvants confers a high level of protection against *Fasciola hepatica* infection in sheep. *Vaccine* **29**(48)**,** 9057-63.

Matsui, M., Fowler, J. H., and Walling, L. L. (2006). Leucine aminopeptidases: diversity in structure and function. *Biol Chem* **387**(12)**,** 1535-44.

McCarthy, E., Stack, C., Donnelly, S. M., Doyle, S., Mann, V. H., Brindley, P. J., Stewart, M., Day, T. A., Maule, A. G., and Dalton, J. P. (2004). Leucine aminopeptidase of the human blood flukes, *Schistosoma mansoni* and *Schistosoma japonicum*. *Int J Parasitol* **34**(6)**,** 703-14.

Ouaissi, M. A., Auriault, C., Santoro, F., and Capron, A. (1981). Interaction between *Schistosoma mansoni* and the complement system: role of IgG Fc peptides in

the activation of the classical pathway by schistosomula. *J Immunol* **127**(4)**,** 1556-9.

Rinaldi, G., Morales, M. E., Alrefaei, Y. N., Cancela, M., Castillo, E., Dalton, J. P., Tort, J. F., and Brindley, P. J. (2009). RNA interference targeting leucine aminopeptidase blocks hatching of *Schistosoma mansoni* eggs. *Mol Biochem Parasitol* **167**(2)**,** 118-26.

Sirisinha, S., Tuti, S., Tawatsin, A., Vichasri, S., Upatham, E. S., and Bunnag, D. (1983a). Attempts to induce protective immunity in hamsters against infection by a liver fluke of man (*Opisthorchis viverrini*). *Parasitology* **86 (Pt 1),** 127-36.

Sirisinha, S., Tuti, S., Vichasri, S., and Tawatsin, A. (1983b). Humoral immune responses in hamsters infected with *Opisthorchis viverrini*. *Southeast Asian J Trop Med Public Health* **14**(2)**,** 243-51.

Smout, M. J., Laha, T., Mulvenna, J., Sripa, B., Suttiprapa, S., Jones, A., Brindley, P. J., and Loukas, A. (2009). A granulin-like growth factor secreted by the carcinogenic liver fluke, *Opisthorchis viverrini*, promotes proliferation of host cells. *PLoS Pathog* **5**(10)**,** e1000611.

Soukhathammavong, P., Odermatt, P., Sayasone, S., Vonghachack, Y., Vounatsou, P., Hatz, C., Akkhavong, K., and Keiser, J. (2011). Efficacy and safety of mefloquine, artesunate, mefloquine-artesunate, tribendimidine, and praziquantel in patients with *Opisthorchis viverrini*: a randomised, exploratory, open-label, phase 2 trial. *Lancet Infect Dis* **11**(2)**,** 110-8.

Sripa, B., Kaewkes, S., Sithithaworn, P., Mairiang, E., Laha, T., Smout, M., Pairojkul, C., Bhudhisawasdi, V., Tesana, S., Thinkamrop, B., Bethony, J. M., Loukas, A., and Brindley, P. J. (2007). Liver fluke induces cholangiocarcinoma. *PLoS Med* **4**(7)**,** e201.

Suttiprapa, S., Mulvenna, J., Huong, N. T., Pearson, M. S., Brindley, P. J., Laha, T., Wongkham, S., Kaewkes, S., Sripa, B., and Loukas, A. (2009). Ov-APR-1, an aspartic protease from the carcinogenic liver fluke, *Opisthorchis viverrini*: functional expression, immunolocalization and subsite specificity. *Int J Biochem Cell Biol* **41**(5)**,** 1148-56.

van den Enden, E. (2009). Pharmacotherapy of helminth infection. *Expert Opin Pharmacother* **10**(3)**,** 435-51.

Yu, J., Chen, T., Xie, Z., Liang, P., Qu, H., Shang, M., Mao, Q., Ning, D., Tang, Z., Shi, M., Zhou, L., Huang, Y., and Yu, X. (2015). Oral delivery of *Bacillus subtilis* spore expressing enolase of *Clonorchis sinensis* in rat model: induce systemic and local mucosal immune responses and has no side effect on liver function. *Parasitol Res*.